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(54) Title: METHOD AND KIT FOR DETECTING MEMBRANE PROTEIN - PROTEIN INTERACTIONS

(57) Abstract: The present invention is concerned with a method and a kit for detecting an interaction between a first membrane bound test protein or fragment thereof and a second test protein or fragment thereof which is either membrane bound or soluble with an *in vivo* genetic system based in yeast, bacterial or mammalian cells. The system makes use of the reconstitution of the split ubiquitin protein.

WO 02/27020 A1

METHOD AND KIT FOR DETECTING MEMBRANE PROTEIN - PROTEIN INTERACTIONS

The present invention is concerned with a method for detecting membrane protein - protein interactions with an *in vivo* genetic system based in yeast, bacterial or mammalian cells. Furthermore, the invention provides a kit for detecting the interactions between a first membrane test protein and a second test protein using reconstitution of the split ubiquitin protein. The reconstitution of split ubiquitin makes use of chimeric genes, which express hybrid proteins.

Background of the Invention

An important area in biology is the analysis of interactions between proteins. Proteins are complex macromolecules made up of covalently linked chains of amino acids. Each protein assumes a unique three-dimensional shape principally determined by its sequence of amino acids. Many proteins consist of smaller units called domains, which are continuous stretches of amino acids able to fold independently from the rest of the protein (e.g. α-helices, β-sheets).

Interactions between proteins mediate most processes in a living cell. They are involved, for example, in the assembly of enzyme subunits, antigen-antibody reaction, in forming the su-

pramolecular structures of ribosomes, filaments and viruses. A special and specific role can be attributed to membrane proteins. They are involved in the transport of molecules; in the interaction of receptors on the cell surface with growth factors and hormones; membrane bound oncogene products can give rise to neoplastic transformation through protein-protein interactions with proteins called kinases whose enzymatic activity on cellular target proteins leads to a cancerous state. Other examples of a protein-protein interaction in membranes occur when virus infects a cell by recognizing a protein (receptor) on the surface, and this interaction has been used to design antiviral agents. There are two types of transmembrane proteins: Type I transmembrane proteins have their C-terminus in the cytoplasm, whereas the Type II transmembrane proteins have their C-terminus outside the cell (or in the inner part of some other organelle, for example in the lumen of the endoplasmic reticulum).

Protein-protein interactions have been generally studied in the past ten years by using biochemical techniques such as cross-linking, co-immunoprecipitation and co-fractionation by chromatography. Biochemical methods have the disadvantage that interacting proteins are generally known as bands of a particular mobility on a polyacrylamide gel. To progress from these bands to cloned genes is often a very tedious process.

A genetic system that is capable of rapidly detecting which proteins interact with a known protein, determining which domains of the proteins interact, and providing the genes for the newly identified interacting proteins has been developed in 1989 by Stan Fields and Ok-Kyu Song [Fields, S. and Song, O.-

K., Nature 340, 245-248 (1989)]. Their system, termed yeast two-hybrid system, is based on reconstitution of a transcriptional activator and transcriptional activation of reporter genes. The yeast two-hybrid system is a powerful method in the *in vivo* analysis of the protein-protein interaction, but is naturally limited to the analysis of soluble proteins or soluble domains of membrane proteins, i.e. interactions between integral membrane proteins cannot be studied. In addition, the hybrid proteins are targeted to the nucleus where the interactions take place. Thus, the interactions that are dependent on post-translational modifications that take place within ER, such as glycosylation and disulfide bond formation, may not occur.

The split-ubiquitin system represents an alternative assay for the *in vivo* analysis of protein interactions. It was developed in 1994 by Niels Johnsson and Alexander Varshavsky [Johnsson, N. and Varshavsky, A., Proc. Natl. Acad. Sci. USA 91, 10340-10344 (1994)] for the detection of interactions between soluble proteins (Figure 1). Ubiquitin (Ub) is a 76 amino acid residue, single domain protein that is present in cells either free or covalently linked to other proteins. Ubiquitin plays a role in a number of processes primarily through routes that involve protein degradation. In eukaryotes, newly formed Ub fusions are rapidly cleaved by ubiquitin specific proteases (UBPs) after the last residue of Ub at the Ub-polypeptide junction. The cleavage of a UB fusion by UBPs requires the folded conformation of Ub. When a C-terminal fragment of ubiquitin (Cub) is expressed as a fusion to a reporter protein, the fusion is cleaved only if an N-terminal fragment of Ubiquitin (Nub) is also expressed in the same cell. This reconstitution of native

ubiquitin from its fragments, detectable by the *in vivo* cleavage assay, is not observed with a mutationally altered Nub. However, if Cub and the altered Nub are each linked to polypeptides that interact in vivo, the cleavage of the fusion containing Cub is restored, yielding a generally applicable assay for detecting the protein-protein interactions (Figure 1).

The system was subsequently modified and shown to work with membrane proteins [I. Stagljar et al., Proc. Natl. Acad. Sci. USA 95, p. 5187-5192, 1998)]. Three yeast membrane proteins of the endoplasmic reticulum have been used as a model system. Wbp1p and Ost1p are both subunits of the oligosaccharyl transferase membrane protein complex. The Alg5 protein also localizes to the membrane of the endoplasmic reticulum, but does not interact with the oligosaccharyltransferase. Specific interactions were detected between Wbp1p and Ost1p, but not between Wbp1p and Alg5p. Therefore, the modified split-ubiquitin system works as a detection system for membrane proteins. In contrast to the conventional two-hybrid system, which requires nuclear localization, the interactions are detected at the natural environment of the protein of interest.

However, none of the aforementioned prior art suggests a genetic method that works for the detection of *in vivo* membrane protein - cytosol protein interactions as well as for membrane protein - membrane protein interactions using transcriptional activation as an assay.

It is therefore an object of the present invention to provide a genetic method for detecting *in vivo* protein interactions of membrane proteins with membrane proteins as well as membrane

proteins with cytosolic (soluble) proteins.

A further object of the present invention is a method for the identification of new genes by screening libraries fused to the N-terminal domain of Ubiquitin (Nub).

Another object of this invention is to provide a method by which a multiplicity of proteins, such as those encoded by the entire genome of a cell, can be simultaneously tested for interaction with a known protein.

Yet another object of the present invention is to provide a method which can be used in the design of peptides to be used therapeutically.

A still further object of this invention is to provide a method for testing affinity reagents for protein purification.

The present invention also provides kits for carrying out each of the above mentioned objects.

Summary of the Invention

These and other objects are achieved by the present invention, which provides a method and a kit for detecting interactions between either two membrane proteins or one membrane and one cytosolic protein. The reconstitution of split ubiquitin makes use of chimeric genes, which express hybrid proteins. Two types of hybrid proteins are prepared. The first hybrid contains a membrane protein of interest (bait) fused to the Cub-PLV module (containing C-terminal domain of ubiquitin (Cub) followed by an

artificial transcriptional activator (ProteinA-LexA-VP16)). The second hybrid protein (prey) contains an N-terminal domain of ubiquitin fused to the second test protein. The prey protein can be either a membrane protein or a soluble cytoplasmic protein. If two test proteins are able to interact, they reconstitute two separate ubiquitin domains into an active ubiquitin leading to the cleavage of the transcriptional activator and activation of the yeast reporter system.

One advantage of this method is that a multiplicity of membrane proteins can be simultaneously tested to determine whether any interact with a known protein. For example, a DNA fragment encoding the membrane protein of interest (bait) is fused to a DNA fragment encoding the Cub-Protein A-LexA-VP16 fusion. This hybrid is introduced into the host cell (yeast, bacterial or mammalian cells) carrying marker genes. For the second partner, a library of plasmids can be constructed which may include, for example, total human complementary DNA (cDNA) fused to the DNA sequence encoding the N-terminal domain of Ubiquitin (NubG). This library is introduced into the yeast cells carrying bait protein. If any individual plasmid from the library encodes a protein that is capable of interacting with the membrane bait protein, a positive signal will be obtained. In addition, when an interaction between proteins occurs, the gene for the newly identified protein is available.

The system can be of value in the identification of new genes. For example, membrane bound receptors may be identified that interact with a known membrane protein. Proteins that interact with oncogene-encoded membrane proteins may be discovered, and these proteins will be of therapeutic value.

The system can be used in the design of peptide inhibitors. For example, peptides that interact with membrane bound growth factor receptors can be identified and then tested in other systems for their ability to inhibit the signal transduction. Peptides that bind to bacterial or viral membrane proteins can be identified and then tested in other systems for their ability to inhibit these bacteria or viruses.

The system can be used to test affinity reagents for protein purification. Peptides or protein domains can be identified that interact with the known membrane protein of interest and these may then be used in a purification protocol for the known protein.

Detailed Description of the Invention

A method for detecting the interaction between a first test membrane protein and a second test (membrane or soluble) protein is provided in accordance to the present invention. The method is set up in yeast, preferably in Schizosaccharomyces pombe, most preferably in the budding yeast Saccharomyces cerevisiae, but can be set up as well in bacteria such as Escherichia coli and mammalian cell systems. The host cell contains a detectable gene having a binding site for a transcriptional activator, preferably PLV (for Protein A-LexA-VP16), such that the detectable gene expresses a detectable protein when the detectable gene is transcriptionally activated.

The first chimeric gene is provided which is capable of being expressed in the host cell. The first chimeric gene contains a

DNA coding for a first test membrane protein fused to the transcriptional activator called PLV (for Protein A-LexA-VP16). This protein is then tested for interaction with a second test protein or protein fragment.

A second chimeric gene is provided which is capable of being expressed in the host cell. The second chimeric gene contains a DNA sequence that encodes a second hybrid protein. The second hybrid protein contains an N-terminal domain of Ubiquitin (NubG). The second hybrid protein also contains a second test protein or a protein fragment which is to be tested for interaction with the first test protein or protein fragment. The second hybrid protein may be encoded in a library of plasmids that contain genomic, cDNA or synthetically generated DNA sequences fused to the DNA sequence encoding the N-terminal domain of Ubiquitin (NubG). The interaction between the first test membrane protein and the second test protein in the host cell, therefore, causes the cleavage of the transcriptional activator that activates transcription of the reporter genes. The method is carried out by introducing the first chimeric gene and the second chimeric gene into the yeast reporter strain. The host cell is subjected to conditions under which the first membrane test protein and the second test protein are expressed in sufficient quantity for the reporter gene to be activated. The cells are then tested for their expression of the detectable gene to a greater degree than in the absence of an interaction between the first test protein and the second test protein.

In this way interactions between a first membrane test protein and a library of proteins can be tested. For example, the first

test membrane protein may be derived from a bacterial membrane protein, a viral membrane protein, an oncogene-encoded membrane protein, a growth-factor receptor or any eukaryotic membrane protein. The second test protein may be derived from a library of plasmids as described above.

The method of the present invention may be practiced using a kit for detecting interaction between a first test membrane protein and a second test protein. The kit includes a container, six vectors and a host cell. The vectors for the membrane based yeast two-hybrid system are schematically shown in Figure 2. The first vector (pY-Cub-PLV), that allows to assay any Type I transmembrane protein (Y), contains a weak yeast promoter selected from the group consisting of the ADH promoter, Cycl promoter and TEF promoter, followed by the unique restriction sites for inserting a DNA sequence encoding a test membrane protein in such a manner that the first test protein is expressed as a fusion to the Cub-Protein A-LexA-VP16 portion. The first vector also contains a terminator sequence which is necessary to terminate the transcription of a given test membrane protein. The first vector does not include the sequence that allows its replication in yeast. This vector is an integrative vector that has to be stable integrated in the yeast genome. Also included on the first vector is a first marker gene (LEU2), the expression of which in the host cell permits selection of cells containing the first marker gene from cells that do not contain the first marker gene.

The second vector (pPLV-Cub-Y), that allows to assay any Type II transmembrane protein (Y), contains a weak yeast promoter selected from the group consisting of the ADH promoter, Cycl

promoter and TEF promoter, followed by the unique restriction site for inserting a DNA sequence encoding a test membrane protein in such a manner that the first test protein is expressed as a fusion to Protein A-LexA-VP16-Cub portion (Figure 2). Note that there is an inverted orientation of the transcription factor fused to the PLV portion. The second vector also contains a terminator sequence which is necessary to terminate the transcription of a given test membrane protein. The second vector does not include the sequence that allows its replication in yeast. As the first vector, this vector is an integrative vector that has to be stable integrated in the yeast genome. Also included on the second vector is a marker gene (LEU2), the expression of which in the host cell permits selection of cells containing the first marker gene from cells that do not contain the first marker gene.

The third vector (pX-NubG) allows the cloning of the prey protein (X) that may be a transmembrane protein or soluble (cytoplasmic) protein (Figure 2). The test protein may be encoded in a library of plasmids that contain genomic, cDNA or synthetically generated DNA sequences fused to the NubG domain. The third vector also includes a promoter selected from the group consisting of the ADH promoter, Cycl promoter and TEF promoter, and does include a transcription termination signal to direct transcription. It also includes a DNA sequence that encodes the N-terminal domain of Ubiquitin (NubG) and a unique restriction site to insert a DNA sequence encoding the second test protein or protein fragment into the vector. Thus, the third vector allows the cloning of the test protein as an N-terminal fusion to the NubG domain. The third vector further includes a means for replicating itself in the host cell, i.e. yeast or bacteria. It

also includes a second marker gene (TRP1), the expression of which in the host cell permits selection of cells containing the second marker gene from cells that do not contain the second marker gene.

The fourth vector (pNubG-X) allows the cloning of the prey protein (X), a transmembrane protein or soluble (cytoplasmic) protein, as a C-terminal fusion to the NubG domain (Figure 2). The fourth vector also includes a promoter selected from the group consisting of the ADH promoter, Cycl promoter and TEF promoter, and transcription termination signal to direct transcription. It also includes a DNA sequence that encodes the N-terminal domain of ubiquitin (NubG) and a unique restriction site to insert a DNA sequence encoding the second test protein or protein fragment into the vector. The fourth vector further includes a means for replicating itself in the host cell and in bacteria. It also includes a second marker gene (TRP1), the expression of which in the host cell permits selection of cells containing the second marker gene from cells that do not contain the second marker gene.

The fifth (pNubI-X) and the sixth (pX-NubI) vector serve as the control vectors of the membrane based yeast two-hybrid system. They are identical to the pNubG-X and pX-NubG vectors, respectively, with the only difference that they contain the wild type Nub domain (NubI). Thus, any in frame fusion of a second test protein to these two vectors will result in a positive signal using the yeast host cell.

While vectors published by Stagljar et al. [Proc. Natl. Acad. Sci. USA 95, 5187-5192 (1998)] enabled the expression of the

yeast Wbp1p, Ost1p and Alg5p only, the newly designed vectors enable now the cloning of any desirable membrane "bait" protein into pY-Cub-PLV and pPLV-Cub-Y, and any desirable "prey" protein or a genomic/cDNA library into pNubG-X or pX-NubG vectors. For easy immunological detection pNubG-X and pX-NubG were tagged with a haemaglutinine(HA) epitope. All vectors were confirmed by DNA sequencing.

The kit includes a host cell, a yeast or bacterial strain that contains the detectable gene having binding sites for the artificial transcription factor Protein A-Cub-PLV. The binding site is positioned so that the reporter gene expresses a reporter protein when two proteins interact in this system. The host cell, by itself, is incapable of expressing a protein having a function of the first marker gene (LEU2), the second marker gene (TRP1), the Cub-PLV portion, or the NubG domain.

The basic strategy of the testing method is shown in Figure 1. The method is based on the previously developed split-ubiquitin technique. The split-ubiquitin technique is based on the ability of Nub and Cub, the N- and C-terminal halves of Ub, to assemble into quasi-native Ubiquitin. Ubiquitin-specific proteases (UBPs), which are present in all eukaryotic cells, recognize the reconstituted Ubiquitin, but not its halves, and cleave the Ubiquitin moiety off a reporter protein that had been linked to the C terminus of Cub. Quite in analogy to the two-hybrid system, the liberation of the reporter serves as a readout indicating the reconstruction of Ubiquitin. The assay is designed in a way that prevents efficient association of Nub and Cub by themselves, but allows it if the two Ubiquitin halves are separately linked to proteins that interact in vivo.

Figure 3a schematically illustrates two test membrane proteins, Y and X. The first chimeric protein contains the Type I transmembrane bait protein Y fused to the Cub-PLV portion resulting in Y-Cub-PLV protein. The second chimeric protein contains a second interacting protein X (here depicted as a membrane protein) fused to the NubG domain called X-NubG. Neither of these proteins, Y-Cub-PLV and X-NubG, is able to activate transcription. The interaction of proteins Y and X, as illustrated in Figure 3b, results in formation of the split-ubiquitin heterodimer. The heterodimer is recognized and cleaved by the Ubiquitin specific proteases (UBPs) (open scissors), liberating PLV. The PLV can enter the nucleus by diffusion and bind to the LexA-binding sites leading to activation of transcription of the lacZ and HIS3 reporter genes. This results in blue cells in the presence of X-gal and growth of the cells on agar plates lacking histidine.

Figure 3c schematically illustrates interaction between a Type II transmembrane protein (Y) and a cytoplasmic protein (X). The first chimeric protein contains the bait protein Y fused to the PLV-Cub portion resulting in PLV-Cub-Y protein. The second chimeric protein contains a second interacting protein X (here depicted as a cytoplasmic protein) fused to the NubG domain called NubG-X. Neither of these proteins, PLV-Cub-Y and NubG-X, is able to activate transcription. The interaction of proteins Y and X, as illustrated in Figure 3c, results in formation of the split-ubiquitin heterodimer. The heterodimer is recognized and cleaved by the Ubiquitin specific proteases (UBPs) (open scissors), liberating PLV-Cub. The PLV-Cub can enter the nucleus by diffusion and bind to the LexA-binding sites leading

to activation of transcription of the lacZ and HIS3 reporter genes. This results in blue cells in the presence of X-gal and growth of the cells on agar plates lacking histidine.

The system is dependent on a number of conditions to properly carry out the method of this invention. The first interacting („bait") protein must be a membrane protein. The bait protein carrying Cub-PLV may not be overexpressed since overexpression would result in false-positives. We found that a soluble Cub-PLV results in gene activation without the need for any Nub. Therefore, the bait fusion protein has to be anchored to the lipid bilayer in order to test for interactions. Soluble proteins of interest might be tested by fusing them to a membrane protein anchor. In addition, the Cub-PLV and NubG domains must be located in the cytoplasm, otherwise the cleavage of the PLV portion cannot occur since UBPs are located only in the cytoplasm of the yeast cell.

Examples

In order to test for interactions between two known proteins or one known protein (bait) and a protein(s) encoded by a cDNA library fragment, Cub-Protein A-LexA-VP16 reporter protein is fused to the C-terminal tail of the target bait transmembrane protein (Y), resulting in the bait construct Y-Cub-PLV. The other candidate protein, the "prey" (X), is fused to the NubG as either a N- or C- terminal fusion creating NubG-X or X-NubG. The bait plasmid encoding Y-Cub-PLV is linearized and integrated into the yeast genome LEU2 locus following the transformation of the "prey" construct (X-NubG or NubG-X). Transfor-

mants are selected and assayed for the production of β-galactosidase. The expression of β-galactosidase indicates that the two hybrid proteins interact and reconstitute a functional ubiquitin molecule.

Example 1

Testing for an interaction between Y and X

1. Construction of two fusion genes.

One is the fusion between a transmembrane protein Y and Cub-PLV (Y-Cub-PLV). The other is a fusion between a known protein X or cDNA library and NubG (X-NubG or NubG-X). The orientation and the reading frame between the two parts of each fusion must be maintained so that the hybrid proteins containing the Y fused to the Cub-PLV portion and X fused to the NubG portion will be expressed (Example 2). As a control for expression and the general topology, NubI fusions to the protein of interest are constructed. NubA has an intermediate affinity to Cub, which may be useful under certain conditions. It has to made sure that the ubiquitin fusions localize topologically to the cytoplasm, since ubiquitin and ubiquitin-specific proteases are present only in the cytoplasm.

2. Transformation of the yeast reporter strain L40 with the linearized bait construct expressing the Y-Cub-PLV fusion. Transformed cells are selected on the appropriate selective plates (dropout plates omitting leucine). This strain is transformed with the "prey" plasmid (X-NubG or NubG-X) or a cDNA library fused to NubG (Example 2). The transformed cells are plated on dropout media lacking uracil and leucine and incubated at 30°C.

3. After colonies appear, the transformants for β-galactosidase activity are tested. The transformants can be streaked out in the form of patches and tested using the filter test assay (Example 3). Transformants expressing NubI/NubA or relevant interacting proteins will turn blue after the filter test assay. The β-galactosidase activity can be quantified by using the liquid assay (Example 4).

4. Another control experiment is the analysis of the interaction mediated cleavage of the PLV portion *in vivo* by Western blot analysis and probing with peroxidase-IgG (Example 5).

Example 2.

Construction of hybrid genes and their transformation in the reporter strain.

All plasmids to be used in the membrane protein applicable two-hybrid system are shuttle vectors, which replicate autonomously in both E.coli and S. cerevisiae. The available vectors and yeast strains are published in Stagljar, I. et al., Proc. Natl. Acad. Sci. USA, 95, pp. 5187-5192 (1998). It is advisable to integrate the fusion gene encoding the bait protein into the chromosome. Expression of the protein fusion from episomal or CEN/ARS plasmids might result in overexpression and false positives.

Example 3.

Filter assay for the detection of β-galactosidase activity.

1. The yeast expressing Y-Cub-PLV are grown together with Nub-fusion proteins for two days at 30°C on sterile Whatman filters on drop-out agar plates lacking leucine and tryptophan. The drop-out-medium is used because cells tend to grow poorly in standard minimal medium.

2. Using forceps, the filter is transferred and dipped into liquid nitrogen for 3 min and allowed to thaw at room temperature.

3. The filters are overlaid with 1.5% agarose in 0.1 M $NaPO_4$-buffer (pH 7.0) containing 0.4 mg/ml X-gal.

4. The filters are incubated at 30°C for 0.4-24 hours.

Example 4.

Quantitation of β-galactosidase activity.

1. Yeast transformants expressing Y-Cub-PLV are inoculated together with Nub-fusion proteins into 3 ml of liquid drop-out medium lacking uracil, leucine and tryptophan.

2. Incubation at 30°C until cultures reach midlog phase (OD_{546} ~1.0)

3. Cells are pelleted from 1 ml of culture, washed once in Z buffer, and resuspend in 300 µl Z-buffer.

4. 100 µl cells are taken and lysed by 3 freeze/thaw cycles.

5. 700 µl Z-buffer containing 0.27 % (v/v) β-mercaptoethanol and 160 µl ONPG (4 mg/ml in Z-buffer) are added and incubated for 1-20 hours at 30°C.

6. 400 µl 0.1 M $NaCO_3$ are added, the samples centrifuged, and the OD_{420} is measured.

7. The β-galactosidase activity is calculated using the formula:

$$\beta\text{-galactosidase units} = 1000 \times OD_{420} / (OD_{546} \times \text{min})$$

Example 5.

Western blot analysis of cells expressing Y-Cub-PLV together with Nub-fusions

1. Yeast cells expressing Y-Cub-PLV together with Nub-fusion proteins are grown at 30°C to an OD_{546} of 0.3-1.2 in drop-out liquid medium lacking leucine and tryptophan.

2. The cells are pelleted and resuspended in 50 µl 1.85 M NaOH per 3 OD units of cells, and incubated on ice for 10 min.

3. The same volume of 50 % trichloroacetic acid is added, and the proteins are precipitated by centrifugation for 5 min.

4. The pellet is resuspended in 50 µl of SDS-sample buffer containing 8 M urea.

5. 20 µl of 1 M Tris.base is added and the protein is dissolved at 37°C (heating to 95°C sometimes results in the clumping of membrane proteins).

6. The samples are centrifuged for 2 min. and 10 µl extract is used for SDS-PAGE/Western blotting analysis.

7. The amount of protein loaded by Coomassie staining of the SDS-gels is verified.

8. The membranes are probed with peroxidase-IgG at 1:5000 dilution. Protein A-fusion proteins are detected by enhanced chemiluminescence (Pierce of Amersham).

Claims

1. A method for detecting an interaction between a first membrane bound test protein or fragment thereof and a second test protein or fragment thereof which is either membrane bound or soluble, the method comprising:

(a) providing a host cell containing a detectable gene having a binding site for a transcriptional activator, such that the detectable gene expresses a detectable protein when the detectable gene is transcriptionally activated;

(b) providing a first chimeric gene capable of being expressed in said host cell, the first chimeric gene coding for a first test membrane protein attached to the DNA-sequence of a first module of a protein responsible for intracellular degradation which again is fused to a transcriptional activator, said first test protein is to be tested with a second protein or protein fragment thereof;

(c) providing a second chimeric gene capable of being expressed in said host cell, the second chimeric gene coding for a second test protein which is either membrane bound or soluble and is attached to the DNA-sequence of a second module of a protein responsible for intracellular degradation;

(d) introducing the first chimeric gene and the second chimeric gene into the host cell enabling an interaction between the first and the second test protein which leads to an interaction between the module of the intracellular degradation protein which in turn leads to the cleavage of the transcriptional activator;

(e) determining whether the detectable gene of the host cell has been activated by the transcriptional activator.

2. The method according to claim 1, wherein the host cell is a yeast, a bacterial or a mammalian cell.

3. The method according to claim 2, wherein the host cell is a Saccharomyces pombe cell or more preferably cells of the budding yeast Saccharomyces cerevisiae.

4. The method according to claim 1 wherein the detectable gene is activated by a natural or artificial activator.

5. The method according to claim 4 wherein the detectable gene is activated by the artificial transcriptional activator protein A-LexA-V16 (PVL).

6. The method according to claim 1 wherein the intracellular degradation protein is ubiquitin comprising a first C-terminal module Cub and a second N-terminal module Nub.

7. The method according to claim 1 wherein the DNA-sequences coding for the first test protein are selected from a group consisting of any bacterial membrane protein, any viral membrane protein, any oncogene-encoded membrane protein, any growth factor receptor or any eukaryotic membrane protein.

8. The method according to anyone of the preceding claims wherein the second test membrane protein comprises a library of plasmids.

9. A kit for detecting binding between a first membrane bound test protein and a second test protein or fragments thereof which is either membrane bound or soluble comprising:

(a) a host cell;

(b) a first vector stable integrated in the host cell genome allowing to assay type I transmembrane proteins comprising a plasmid portion, a first gene coding for a membrane bound protein, a module of an intracellular degradation protein, a transcriptional activator, a promoter, unique restriction sites, a transcription termination signal, a first marker gene but no replication initiating sequence;

(c) a second vector stable integrated in the host cell genome allowing to assay type II transmembrane proteins, comprising a plasmid portion, a first gene coding for a membrane bound protein, a first module of an intracellular degradation protein, a transcriptional activator fused in an inverted orientation to the plasmid portion, a promoter, unique restriction sites, a transcription termination signal, a first marker gene but no replication initiating sequence;

(d) a third vector allowing the cloning of the gene encoding the transmembrane or soluble protein comprising a plasmid, said gene which is fused to the N-terminus of a mutationally altered second module of an intracellular degradation protein, a promoter, a transcription termination signal, a unique restriction site, a second marker gene and , means for replication in a host cell;

(e) a fourth vector allowing the cloning of the gene encoding the transmembrane or soluble protein comprising a plasmid, said gene which is fused to the C-terminus of a mutationally altered second module of an intracellular degradation

protein, a promoter, a transcription termination signal, a unique restriction site, a second marker gene and means for replication in a host cell;

(f) a fifth and a sixth vector identical to the third and the fourth vector with the exception of the second module of an intracellular degradation protein, which is wildtype, said vectors serving as control vectors.

10. The kit according to claim 9, wherein the host cell is a yeast, bacterial or mammalian cell.

11. The kit according to claim 10, wherein the host cell is a yeast cell, preferably of Saccharomyces pombe and most preferably of the budding yeast Saccharomyces cerevisiae.

12. The kit according to claim 9 claim wherein the detectable gene is activated by a natural or artificial activator.

13. The kit according to claim 12 wherein the detectable gene is activated by the artificial transcriptional activator Protein A-LexA-V16 (PVL).

14. The kit according to claim 9, wherein the intracellular degradation protein is ubiquitin comprising a first C-terminal module Cub and a second N-terminal module Nub.

15. The kit according to claim 9 wherein the promoters from steps (a) to (f) are selected from the group consisting of ADH promoter, Cyc 1 promoter or TEF1 promoter.

16. The kit according to claim 9 wherein the DNA-sequences

coding for the first test protein are derived from any bacterial membrane protein, any viral membrane protein, any oncogene-encoded membrane protein, any growth factor receptor or any eukyaryotic membrane protein.

17. The kit according to claim 9 wherein the DNA-sequences coding for the second test protein are derived from a library of plamids.

18. Use of the method of claim 1.

19. Use of the kit of claim 9.

Figure 1

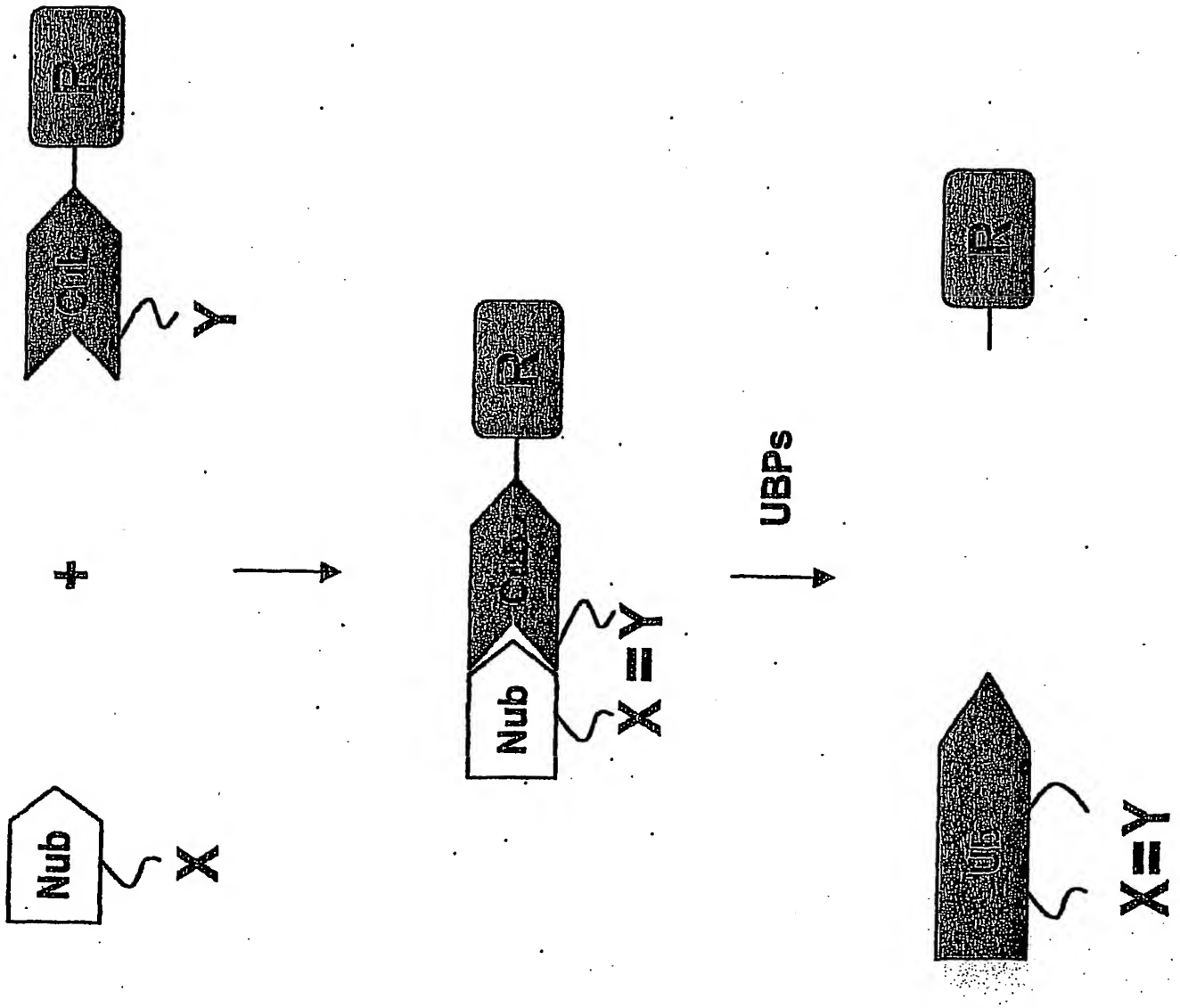


Figure 2

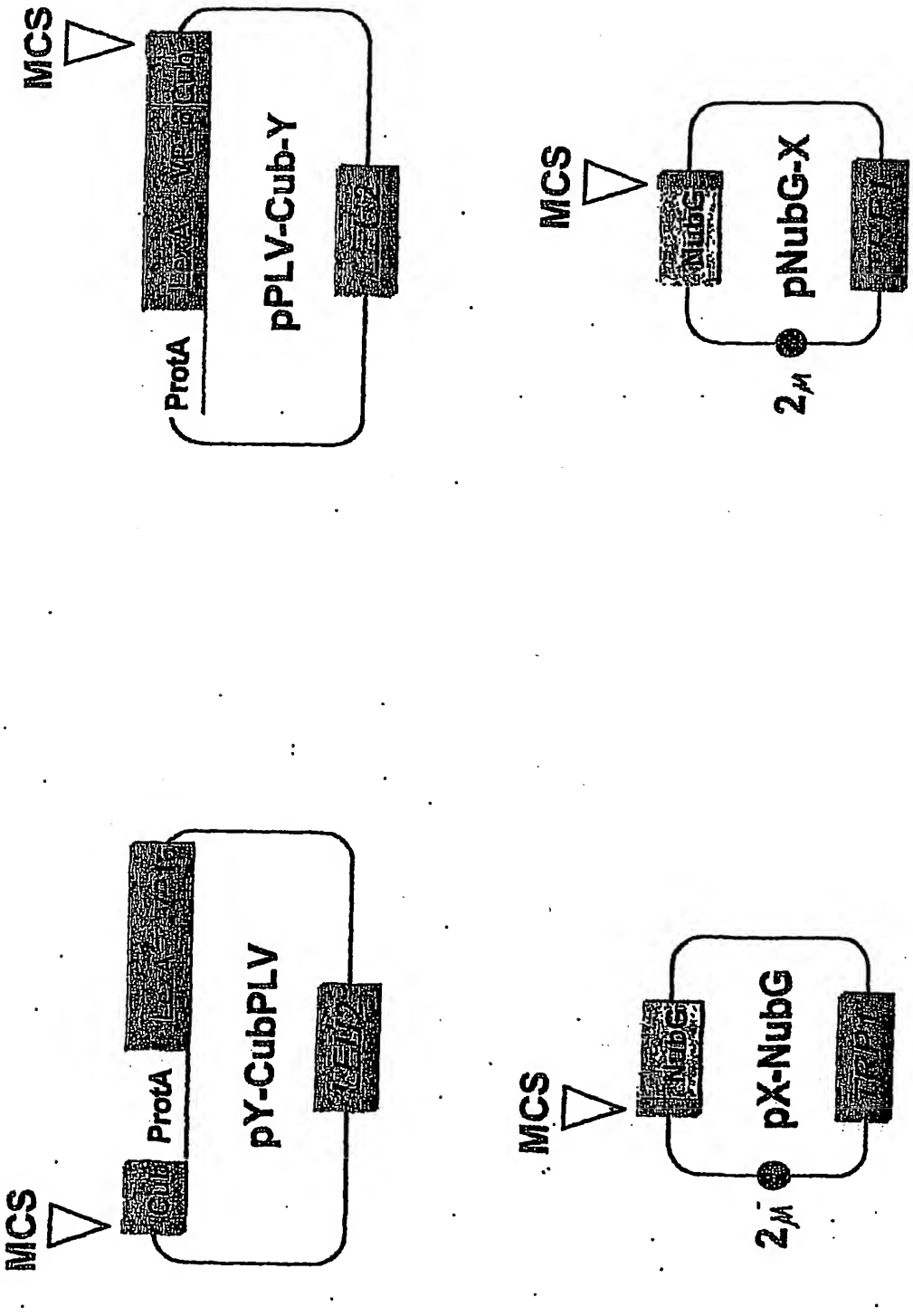
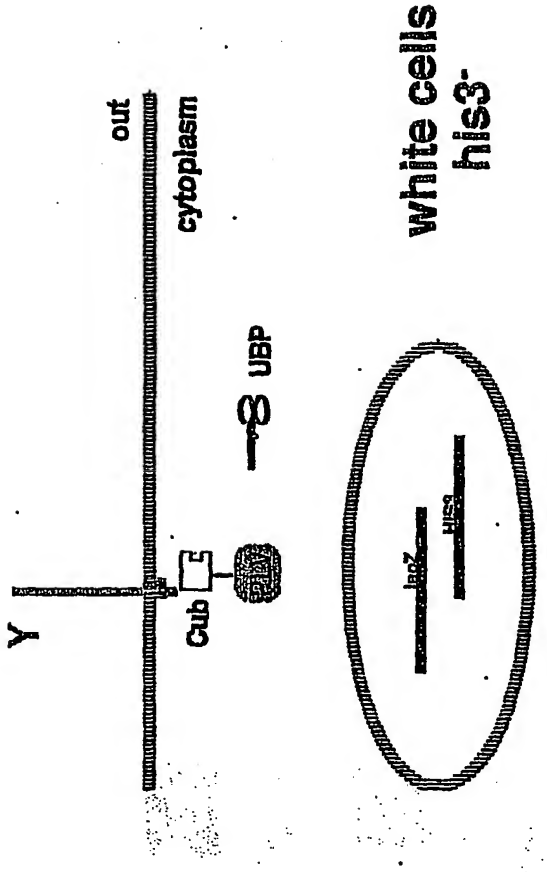

MCS
pPLV-Cub-Y
ProtA
MCS
pNubG-X
2μ
MCS
pY-CubPLV
ProtA
MCS
pX-NubG
2μ

Figure 3a


out
cytoplasm
white cells
his3-
UBP
Cub
Y
lacZ
HIS3

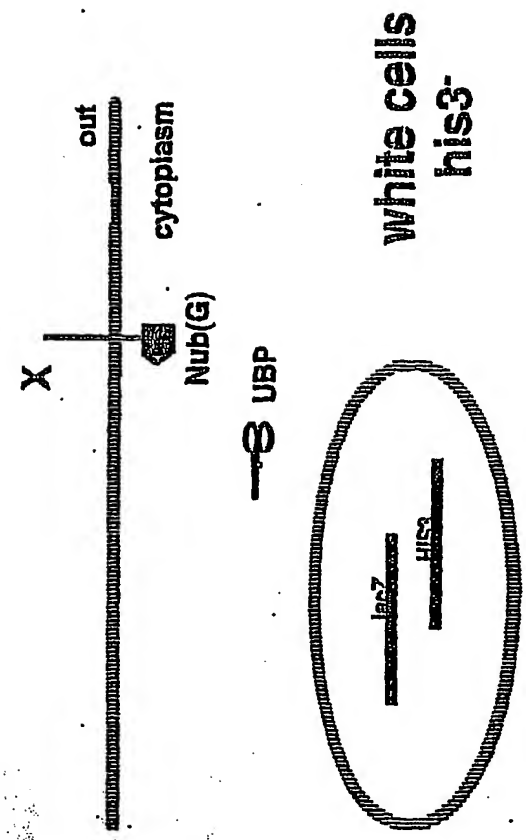

out
cytoplasm
white cells
his3-
X
Nub(G)
UBP
lacZ
HIS3

Figure 3c

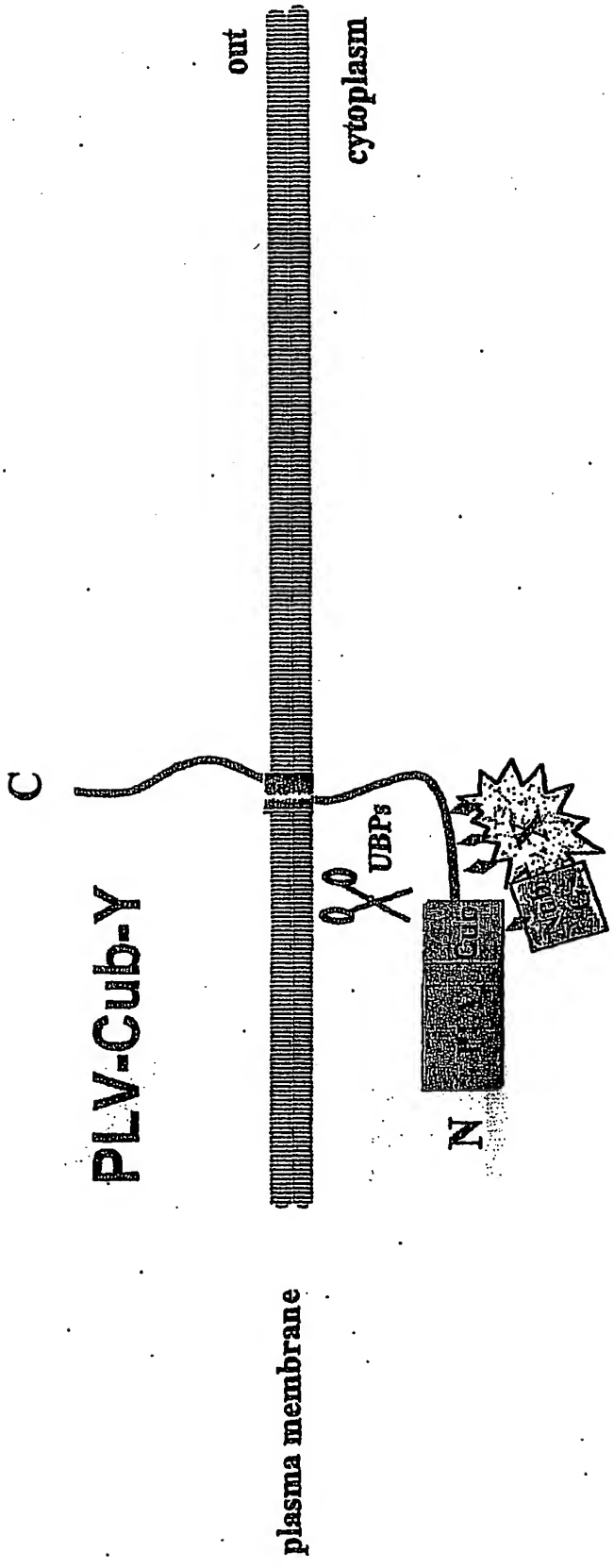
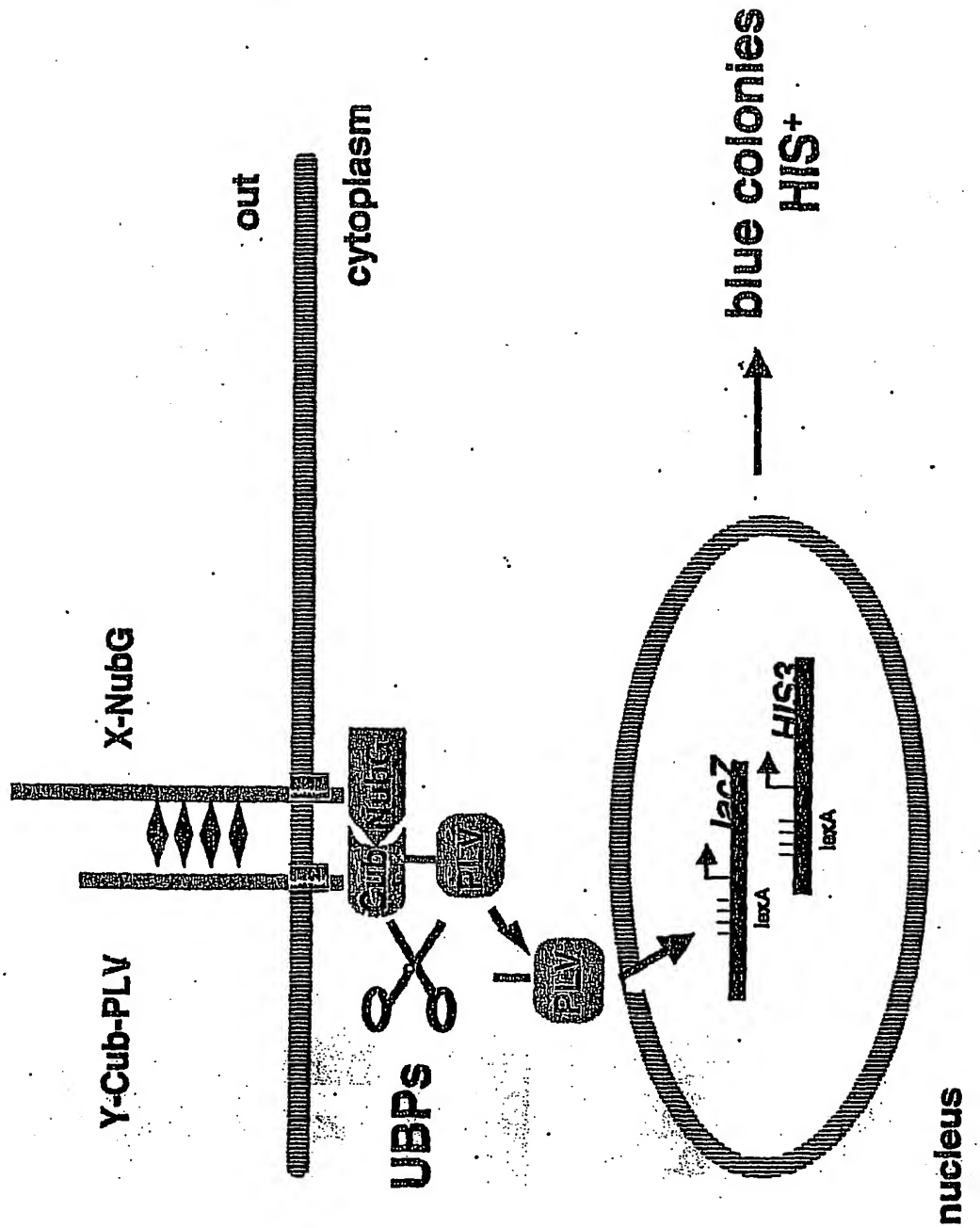


Figure 3b


blue colonies
HIS+
out
cytoplasm
X-NubG
Y-Cub-PLV
UBPs
nucleus
lacZ
HIS3
lexA

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CH [redacted]0534

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 503 977 A (JOHNSSON NILS ET AL) 2 April 1996 (1996-04-02) the whole document	1-3,6, 9-11,14, 18,19
Y	WO 99 55356 A (UNIV YALE) 4 November 1999 (1999-11-04) * see especially claims 1,5,14 and 18 * the whole document	1-3,6, 9-11,14, 18,19
A	FIELDS S ET AL: "A NOVEL GENETIC SYSTEM TO DETECT PROTEIN-PROTEIN INTERACTIONS" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 340, 20 July 1989 (1989-07-20), pages 245-246, XP002018198 ISSN: 0028-0836 cited in the application the whole document	
A	WO 99 12033 A (RUPPERSBERG PETER ;ANTZ CHRISTOF (DE); HERLITZE STEFAN (DE); PAYSA) 11 March 1999 (1999-03-11) the whole document	
A	ALLEN J B ET AL: "Finding prospective partners in the library: the two-hybrid system and phage display find a match" TIBS TRENDS IN BIOCHEMICAL SCIENCES,ELSEVIER PUBLICATION, CAMBRIDGE,EN, vol. 20, no. 12, December 1995 (1995-12), pages 511-516, XP004222355 ISSN: 0968-0004 the whole document	

INTERNATIONAL SEARCH REPORT

International Application No
I[illegible]/CH [illegible]00534

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STAGLJAR I ET AL.: "A genetic system based on split-uniquitin for the analysis of interactions between membrane proteins in vivo" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 95, April 1998 (1998-04), pages 5187-5192, XP002171779 cited in the application the whole document	1-19
X	STAGLJAR I AND TE HEESEN S: "Detecting interactions between membrane proteins in vivo using chimeras. In: Methods in Enzymology: Applications of chimeric genes and hybrid proteins, Part B" 2000 , ACADEMIC PRESS XP002171780 327 page 190 -page 198	1-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 July 2001	24/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

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Ii ation[redacted]ication No
PCT/CH 00/00534

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WO 9955356 A	04-11-1999	AU 3663199 A	16-11-1999
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